

RESEARCH ARTICLE

Propolis suppresses atopic dermatitis through targeting the MKK4 pathway

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Abstract

Propolis is a natural resinous substance made by bees through mixing various plant sources. Propolis has been widely recognized as a functional food due to its diverse range of beneficial bioactivities. However, the therapeutic effects of consuming propolis against atopic dermatitis (AD) remain largely unknown. The current study aimed to investigate the potential efficacy of propolis against AD and explore the active compound as well as the direct molecular target. In HaCaT keratinocytes, propolis inhibited TNF- α -induced interleukin (IL)-6 and IL-8 secretion. It also led to a reduction in chemokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage-derived chemokine (MDC), while restoring the levels of barrier proteins, filaggrin and involucrin. Propolis exhibited similar effects in AD-like human skin, leading to the suppression of AD markers and the restoration of barrier proteins. In DNCB-induced mice, oral administration of propolis attenuated AD symptoms, improved barrier function, and reduced scratching frequency and transepidermal water loss (TEWL). In addition, propolis reversed the mRNA levels of AD-related markers in mouse dorsal skin. These effects were attributed to caffeic acid phenethyl ester (CAPE), the active compound identified by comparing major components

Abbreviations: AD, atopic dermatitis; AMP-PNP, Adenylyl imidodiphosphate; CAPE, caffeic acid phenethyl ester; DNCB, 2,4-dinitrochlorobenzene; ELISA, enzyme-linked immunosorbent assay; IF, immunofluorescence; MCP-1, Monocyte chemoattractant protein-1; MDC, Macrophage-derived chemokine; MKK, Mitogen-activated protein kinase kinase; TEWL, transepidermal water loss; TSLP, thymic stromal lymphopoietin; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

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of propolis. Mechanistic studies revealed that CAPE as well as propolis could directly and selectively target MKK4. Collectively, these findings demonstrate that propolis may be used as a functional food agent for the treatment of AD.

KEYWORDS

atopic dermatitis, caffeic acid phenethyl ester, MKK4, propolis, skin barrier

1 | INTRODUCTION

Atopic dermatitis (AD) is a chronic skin disease that can affect individuals of all age groups, with an estimated 2%–22% of children worldwide being affected, particularly prevalent in industrialized countries.¹ AD is characterized by inflammation, barrier dysfunction, and persistent pruritus of the skin.² The pathogenesis of AD involves a complex interplay between environmental factors, disturbed skin barrier, abnormal skin microbiome, and immune dysregulation.³ When the skin barrier is disrupted, keratinocytes produce cytokines that activate a type 2 immune response and trigger itchiness.⁴ Activated T helper (Th) type 2 cells secrete Th2 cytokines, further impairing the skin barrier.⁴ The resulting itchiness prompts patients to scratch, intensifying the barrier disruption.⁵ This disrupted barrier renders the skin more vulnerable to the invasion of external allergens, thereby reinforcing immune responses and building a complex feedback loop that worsens AD.

Pruritus (itching) is one of the chief hallmarks of AD and can be intense and debilitating, causing significant distress for those affected.⁶ In AD lesions, immunoregulatory cytokines, including alarmins (e.g., IL-25 and IL-33) and thymic stromal lymphopoietin (TSLP) produced by keratinocytes, can activate immune cells and elevate the allergic signaling cascade.^{7,8} These cytokines are also known to stimulate receptors involved in pruritus, causing itchiness in AD patients.⁸ As AD patients experience itching, they scratch their skin lesions, causing substantial damage to the skin barrier. Therefore, the suppression of alarmins and TSLP can be beneficial in preventing skin damage and the exacerbation of AD.

The skin barrier performs crucial functions in protecting the body from external irritants and preventing water loss. Proteins, such as filaggrin, involucrin, and loricrin, are essential components of the skin barrier and are critical for maintaining the structural integrity of the outermost layer of the epidermis.⁹ Filaggrin, in particular, plays a major role by binding to keratin fibers and aggregating them to form a robust network in the epidermis.¹⁰ Patients with AD suffer reduced expression of filaggrin, and filaggrin mutations occur in 20%–50% of AD patients.¹¹ When the skin barrier is disrupted due to

malfunctioning filaggrin or scratching, it becomes more susceptible to irritation, inflammation, and infection.²

Cutaneous inflammation is a critical factor in the pathogenesis of AD. Lesional skin of AD patients displays a predominant Th2-driven immune dysregulation.¹² Activated Th2 cells secrete type 2 cytokines, such as IL-4 and IL-13, which can directly downregulate barrier proteins and promote epidermal barrier dysfunction.⁵ Alarmin secretion driven by skin barrier dysfunction can trigger the activation of inflammatory epidermal dendritic cells and Th2 responses.¹³ Th2 cytokines can also stimulate IgE class switching in B cells and the production of IgE.¹⁴ In addition, nonlesional AD skin exhibits immune infiltrates that produce inflammatory mediators and a type-2 shifted cytokine profile.¹⁵ Given that inflammation is a central driving factor in AD pathophysiology, anti-inflammatory approaches are considered the basic management strategy for AD patients.

Several treatment options are available to alleviate AD symptoms and improve overall skin health. Topical treatments with moisturizers, glucocorticosteroids, and calcineurin inhibitors help reduce inflammation and relieve pruritus.¹⁶ In some cases, oral treatments such as antihistamines or immunosuppressants may also be prescribed. However, these medications may cause unwanted side effects, including an increased risk of infection, liver toxicity, kidney malfunction, and diabetic complications.^{16–18} Consequently, there is a growing need to discover new therapeutic agents for AD treatment and prevention.

Propolis is a natural substance made by bees using plant resin, wax, and their saliva to protect their nests from microbes. Notably, the content of propolis varies depending on the plants from which it is sourced, leading to regional differences. The properties of propolis are influenced by climatic attributes, and bees collect resources from various plants, resulting in complex and distinct mixtures across different countries.^{19,20} As a result, regional propolis has been reported to have different functions.^{20,21} Nonetheless, some bioactivities of propolis have been reported, including antibacterial, anticancer, and anti-skin aging effects.^{22–24} Some studies have also shown that propolis possesses immunomodulatory functions by upregulating TLR-4 and reducing IL-



10.²⁵ In addition, propolis displayed anti-inflammatory activities in animal models of rheumatoid arthritis and sepsis.^{26,27} However, the therapeutic effect of propolis against AD is largely unknown.

In this study, we investigated the effects of propolis on regulating inflammation, pruritus, and barrier dysfunction using HaCaT keratinocytes, human skin tissues, and DNCB-induced AD-like BALB/c mice. Furthermore, we elucidated the underlying molecular mechanisms and identified the active compounds responsible for the anti-AD activity of propolis.

2 | MATERIALS AND METHODS

2.1 | Materials

Antibodies for the detection of phospho-p38, phospho-ERK, ERK, phospho-JNK, phospho-IKK, IKK, IκB, phospho-NF-κB, NF-κB, phospho-Akt, Akt, phospho-MKK3/MKK6, MKK3, and phospho-MKK4 were acquired from Cell Signaling Technology (Danvers, MA, USA). Antibodies for the detection of Involucrin, Filaggrin, p38, JNK, MKK4, GAPDH, β-actin, and vinculin were acquired from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies for the detection of TSLP and IL-6 were purchased from Abcam (Cambridge, United Kingdom). Antibody to detect Loricrin (LOR; #PA5-30583) was acquired from Invitrogen (Waltham, Massachusetts, USA). Caffeic acid phenethyl ester (CAPE) (CAS No. 104594-70-9) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

2.2 | Preparation of propolis extract

Propolis used in this study was sourced from the Korea Apicultural Cooperative Federation (Yangbong Non-ghyup, Seoul, Republic of Korea) and collected from various regions in Korea during the spring of 2021. To prepare the propolis for experimentation, it was processed into a powdered form and immersed in an 80% ethanol solution. The ethanol-extracted propolis was then dried at 60°C for ethanol evaporation and forming a resin.

2.3 | Cell culture

HaCaT cells were cultured to a confluence of 70%–80% in media mixed by Dulbecco's modified Eagle's medium (DMEM; Welgene, Gyeongsan, Republic of Korea), 1% penicillin/streptomycin (Corning Inc., NY, USA), 10%

fetal bovine serum (FBS; Gibco, NY, USA) with Chelex 100 Resin (Bio-Rad, CA, USA), and 0.07 mM calcium chloride (Welgene) in an incubator maintaining 37°C and a humidified CO₂ incubator with HEPA filter.

2.4 | Enzyme-linked immunosorbent assay (ELISA)

HaCaT cells were seeded in 12-well culture plates (2×10^4 cells/mL) and incubated in an incubator. The next day, the medium used for seeding was changed into DMEM containing 1% FBS for an additional 24 h for starvation. Next, propolis was treated to cells with indicated concentrations. After 1 h, a proinflammatory cytokine (TNF-α) was treated to cells for 24 h. The medium was collected by 13,000 rpm centrifugation. The concentration of cytokines and chemokines in the supernatants was examined with specific DuoSet ELISA kits (R&D Systems Inc., Minneapolis, USA). The result absorbance values were evaluated using the Varioskan™ LUX multi-mode microplate reader (Thermo Fisher Scientific, MA, USA).

2.5 | Quantitative real-time polymerase chain reaction (qRT-PCR)

HaCaT cells were plated in 6-well culture plates, incubated, and then starved with 1% FBS DMEM for 24 h. Then, TNF-α was treated to cells following the pretreatment of propolis. To measure RNA level, total RNA was isolated from HaCaT cells or dorsal skin with RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's experimental procedure. The cDNA was synthesized from 900 ng of total RNA with a ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan). The mRNA expression level was analyzed by examination of SYBR® Green Master Mix (TOYOBO) with CFX Connect Optics Module System (Bio-Rad Laboratories, Inc., California, USA). The primer sequences of each gene were shown in Table S1.

2.6 | Ex vivo human skin model

Skin tissue samples of healthy humans were obtained from Biopredic International (Saint-Grégoire, France). The human skin tissue samples were collected following the French Law CSP1245-2, and the French Research Ministry granted a permit (AC-2013-1754). Tissue samples were collected from the breast of a 20-year-old female human and cultured in DMEM (Welgene) at 37°C

in a 5% CO₂ incubator, in accordance with the supplier's protocol. The DMEM media for the ex vivo human skin tissues was changed into new media containing TNF- α (10 ng/mL) and propolis (5 μ g/mL) every 2 days. The cultured media were collected once every 4 days for conducting ELISA. After 14 days, half of each tissue was stored for immunofluorescence (IF) and the other half for immunoblotting.

2.7 | Immunoblot analysis

Harvested cells or tissue samples were added with RIPA buffer and lysed on ice. The supernatants were obtained by centrifugation at 4°C, 13,000 rpm for 10 min. The concentrations of protein were determined with a BCA assay (Thermo Fisher Scientific). Total proteins (40 μ g) were subjected to and separated by the SDS-PAGE method. The separated proteins were transferred from the gel to BioTrace™ NT nitrocellulose transfer membranes (PALL Corporation, NY, USA). The membrane was blocked with 5% nonfat dry milk in TBS with Tween 20 at room temperature for 1 h and incubated overnight in primary antibody. Next, the membranes were incubated in secondary antibodies for 1 h and WestGlow™ DURA Chemiluminescent substrate (Biomax, Guri, Republic of Korea) was used to visualize the protein bands on the film.

2.8 | Immunofluorescence (IF)

For immunostaining, the human tissue samples were fixed with 4% paraformaldehyde, embedded in paraffin, and permeabilized with 0.3% Triton X-100. The tissues were then blocked in 1% bovine serum albumin (BSA) in PBS with tween 20 for 1 h at room temperature, followed by incubation with the primary antibody detection for filaggrin in 1% BSA in PBST at 4°C overnight. After washing with 1% BSA in PBST, the slides were incubated with a secondary antibody (Alexa Fluor 488) (Thermo Fisher Scientific) for 2 h. The bright field and fluorescence images were visualized and captured using LSM 980 (Carl Zeiss, Oberkochen, Germany).

2.9 | Animals

BALB/c mice (8 weeks old, female) were obtained from Orient Bio (Seongnam, Republic of Korea). The mice were housed in cages maintained under pathogen-free conditions with a temperature of $23 \pm 1.5^\circ\text{C}$, relative humidity of $55\% \pm 15\%$, and a 12-h light–dark cycle. Mice were provided with an approved diet (LabDiet 5 L79, Orient Bio)

and water ad libitum. All animal experimental procedures were conducted in accordance with the protocol of the Institutional Animal Care and Use Committee (IACUC-YS-2022-7) of Chonnam National University.

2.10 | Animal model

The dorsal hair was shaved, and then 4% SDS (150 μ L) was applied. After 3 h, 2,4-dinitrochlorobenzene (DNCB) was applied to the dorsal skin. On the 14th day after induction, the mice were randomly classified into five groups ($n = 8$): (1) Vehicle group (a negative control; PBS sensitization and vehicle applied), (2) AD group (DNCB sensitization and vehicle applied), (3) propolis 40 group (DNCB + propolis 40 mg/kg B.W.), (4) propolis 120 group (DNCB + propolis 120 mg/kg B.W.), and (5) Cetirizine 5 group (a positive control; DNCB + Cetirizine 5 mg/kg B.W.). Propolis extract and cetirizine were orally administered to the mice daily for 3 weeks. At the end of the experiment, the mice were euthanized using isoflurane after their body weights were measured.

2.11 | Transepidermal water loss (TEWL), scratching behavior, and ear thickness measurement

To measure the skin dryness of the dorsal skin of DNCB-stimulated or nonstimulated BALB/c mice, the TEWL was measured once a week by the Tewameter TM300 device. To measure the scratching frequency of DNCB-stimulated or nonstimulated mice, the number of scratching their body for 10 min was counted once a week. To investigate the skin edema on the ear of DNCB-induced mice, the ear thickness of all mice was examined using an electronic caliper (Hornady, GI, USA) before sacrifice at the end of the animal experiment (week 5).

2.12 | Hematoxylin and eosin (H&E) staining

To analyze the dorsal skin histologically, the dorsal skin tissue samples were collected and fixed in 4% paraformaldehyde and embedded in paraffin. Sections of tissues were prepared using a microtome and mounted on slides. The slides were immersed in a hematoxylin solution for 5 min. Following this, the slides were washed and differentiated in acid alcohol. Subsequently, the slides were stained in eosin solution for 2 min to impart the cytoplasmic components. The stained slides were observed using a light microscope for visualization of the skin tissues.

2.13 | Kinase assays

Kinase assays were conducted using the SelectScreen Kinase profiling services (Thermo Fisher Scientific) and the KinaseProfiler services (Eurofins Scientific, Dundee, United Kingdom). The services were carried out following the supplier's experimental procedure for determining the kinase activity with or without propolis or CAPE.

2.14 | UHPLC-MS/MS analysis

The identification of organic compounds in propolis was carried out using the Thermo Vanquish instrument (Thermo Fisher Scientific). The method employed was liquid chromatography-mass spectrometry (LC-MS) with the TSQ Altis Plus mass spectrometer (Thermo Fisher Scientific). Compounds were separated using a CORTECS C18 column (Waters Corporation, Milford, MA, USA). For detection, selected reaction monitoring (SRM) was utilized, and the ionization process employed a heated electrospray ionization (HESI) probe. The constant flow rate was set to 0.3 mL/min, and the volume of sample injection was 1 μ L. The mobile phase consisted of an aqueous phase A (0.1% formic acid) and an organic phase B (methanol, 0.1% formic acid) using a gradient elution process of 10% B, 0–0.1 min; 10–30% B, 0.1–1 min; 30–100% B, 1–10 min; 100% B, 10–11.5 min; 100%–10% B, 11.5–11.6 min; and 10% B, 11.6–15 min. Quantification of the major components in propolis was achieved using calibration curves, shown in Table S2, with each analyzed in triplicate. Quantification chromatograms of each compound are shown in Figure S4.

2.15 | Modeling of CAPE binding complex

The ternary complex of nonphosphorylated MKK4 with Adenylyl imidodiphosphate (AMP-PNP) and the p38 α was obtained from the Protein Data Bank (PDB code: 3ALO).²⁸ CAPE was docked to each of the two active sites of MKK4 using the CDocker docking method, adopting the CHARMM force field in Discovery Studio software.²⁹ For the docking analysis, active sites were defined as spheres with radii of 10.3 Å (for the ATP binding site) and 16.5 Å (for the p38 α binding site), with each sphere centered on its respective ligand. Ligand conformational entropy and in situ minimization were considered to calculate the binding energy of the most reliable binding mode based on the docking results.

2.16 | Statistical analysis

The data are presented as mean $\pm$ standard deviation (SD) or mean $\pm$ standard error of the mean (SEM). Statistical differences of data among groups were tested using unpaired, two-tailed *t*-test, analysis of variance (ANOVA), or Brown-Forsythe and Welch ANOVA followed by Tukey's post hoc test by GraphPad Prism 5 software (San Diego, CA, USA). A statistical significance level was considered as $p < 0.05$.

3 | RESULTS

3.1 | Propolis inhibits atopic dermatitis (AD) cytokine/chemokine production and restores skin barrier proteins in keratinocytes

First, the nontoxic concentration of propolis was evaluated. Propolis showed no cytotoxic effects on HaCaT keratinocytes at concentrations up to 5 μ g/mL (Figure S1). Therefore, the concentration range of 1.25–5 μ g/mL was utilized for subsequent experiments. TNF- α was used to simulate atopic dermatitis (AD) conditions in keratinocytes.^{30,31} Treatment with TNF- α resulted in a significant upregulation of proinflammatory cytokines (IL-6 and IL-8) and chemokines (MCP-1 and MDC) in keratinocytes (Figure 1A–D). Propolis treatment inhibited the production of TNF- α -induced cytokines and chemokines in a dose-dependent manner (Figure 1A–D). Furthermore, TNF- α treatment led to a reduction in skin barrier proteins such as filaggrin and involucrin, but propolis effectively restored their mRNA levels (Figure 1E,F).

3.2 | Propolis suppresses AD-related biomarkers and restores skin barrier proteins in human skin tissue

To further examine whether the anti-AD effects of propolis could be recapitulated in human skin, a human skin-based model was employed. Human skin tissues were incubated with TNF- α for 14 days to induce AD-like skin, and biomarkers were analyzed (Figure 2A). Within the human skin tissues, the expression of pruritus-inducing TSLP and the inflammatory cytokine IL-6 was upregulated by TNF- α treatment. However, propolis treatment lowered TNF- α -mediated TSLP and IL-6 levels (Figure 2B). Skin barrier proteins, filaggrin and involucrin, were downregulated in the TNF- α -induced tissue (Figure 2B). However, the treatment of propolis effectively restored the expression of filaggrin and involucrin

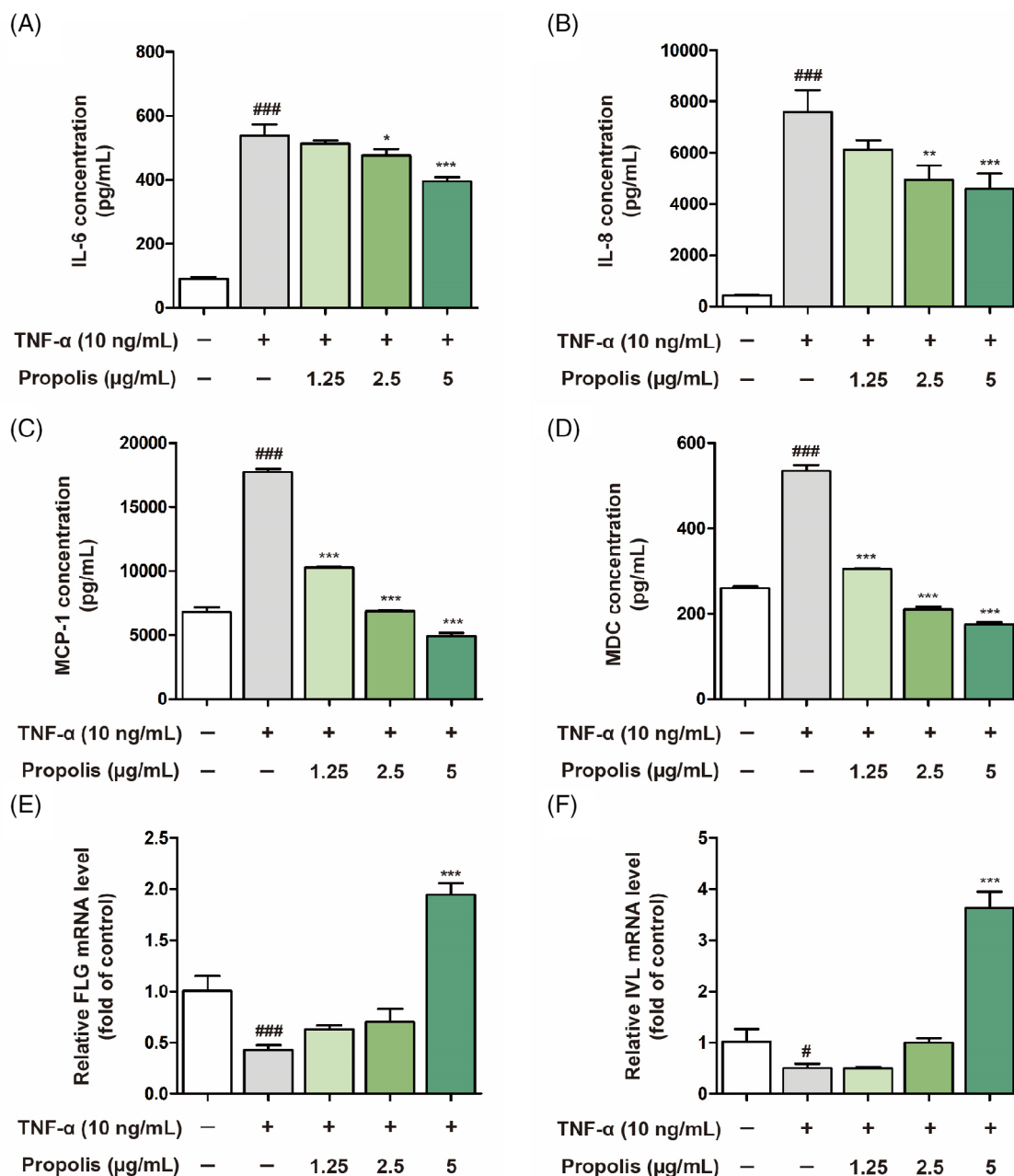


FIGURE 1 Effects of propolis on the level of cytokines, chemokines, and barrier proteins in HaCaT keratinocytes. Production level of cytokines (A) IL-6 and (B) IL-8, and chemokines (C) MCP-1 and (D) MDC in culture media was determined using ELISA ($n = 3$). The expression of skin barrier proteins, (E) FLG and (F) IVL was determined by qRT-PCR. Data represent the mean $\pm$ standard deviation (SD). [#] $p < 0.05$; ^{###} $p < 0.001$ versus control. ^{*} $p < 0.05$; ^{**} $p < 0.01$; ^{***} $p < 0.001$ versus induced group. FLG, filaggrin; IVL, involucrin; MCP-1, monocyte chemoattractant protein-1; MDC, macrophage-derived chemokine; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

(Figure 2B). In the ex vivo tissue-cultured media, while the concentration of IL-6, IL-8, and MCP-1 progressively increased in the TNF- α treated group over time, indicative of AD aggravation, the treatment of propolis significantly inhibited the levels of these proinflammatory proteins (Figure 2C–E). Moreover, we assessed the level of filaggrin using immunofluorescence staining. Filaggrin, shown in green color, is predominantly expressed in

the epidermis of human skin tissue. Upon TNF- α incubation, the level of filaggrin was decreased but was reinstated in the presence of propolis (Figure 2F). Collectively, these results suggest that propolis can suppress the progression of AD by inhibiting factors that induce inflammation and pruritus while simultaneously promoting the recovery of skin barrier proteins in human skin tissues.

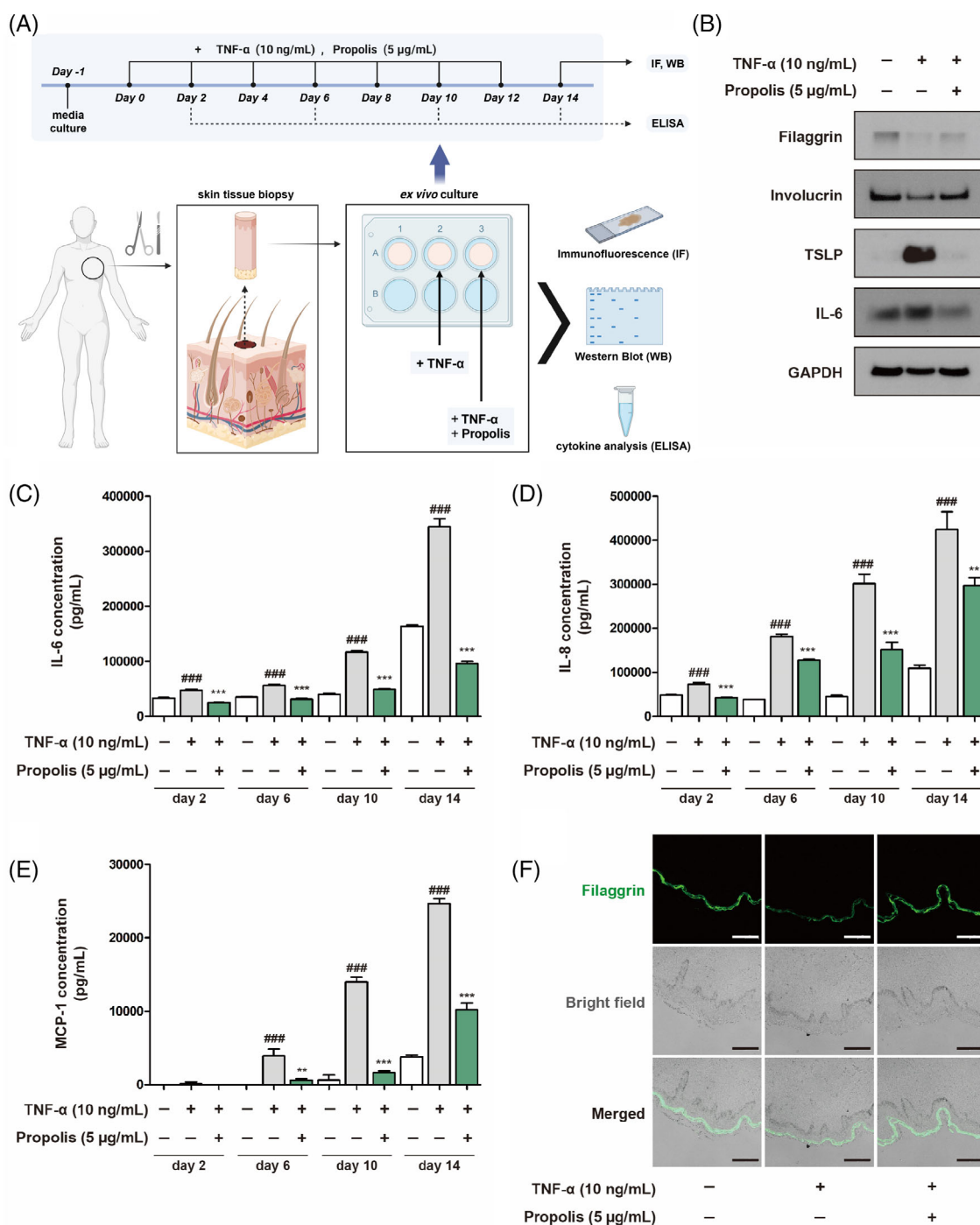


FIGURE 2 Effects of propolis on the levels of AD-related biomarkers and barrier proteins in human skin tissues. (A) Experimental process and timeline of an ex vivo human skin tissue model (created with BioRender.com). (B) The expression of AD-related markers in tissues was determined using western blotting. GAPDH served as the loading control. (C–E) The culture media of skin tissues at days 2, 6, 10, and 14 were collected, and the secretion levels of cytokines (C) IL-6 and (D) IL-8, and chemokine (E) MCP-1 were measured by ELISA ($n = 3$). Data represent the mean $\pm$ standard deviation (SD). $^{###}p < 0.001$ versus control group of each day. $^{**}p < 0.01$; $^{***}p < 0.001$ versus induced group of each day. (F) Expression of filaggrin was detected using immunofluorescence staining (scale bar: 100 μ m). Representative images of filaggrin (green), bright field (gray), and merged images were presented. AD, atopic dermatitis; ELISA, enzyme-linked immunosorbent assay; TSLP, thymic stromal lymphopoietin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MCP-1, monocyte chemoattractant protein-1.

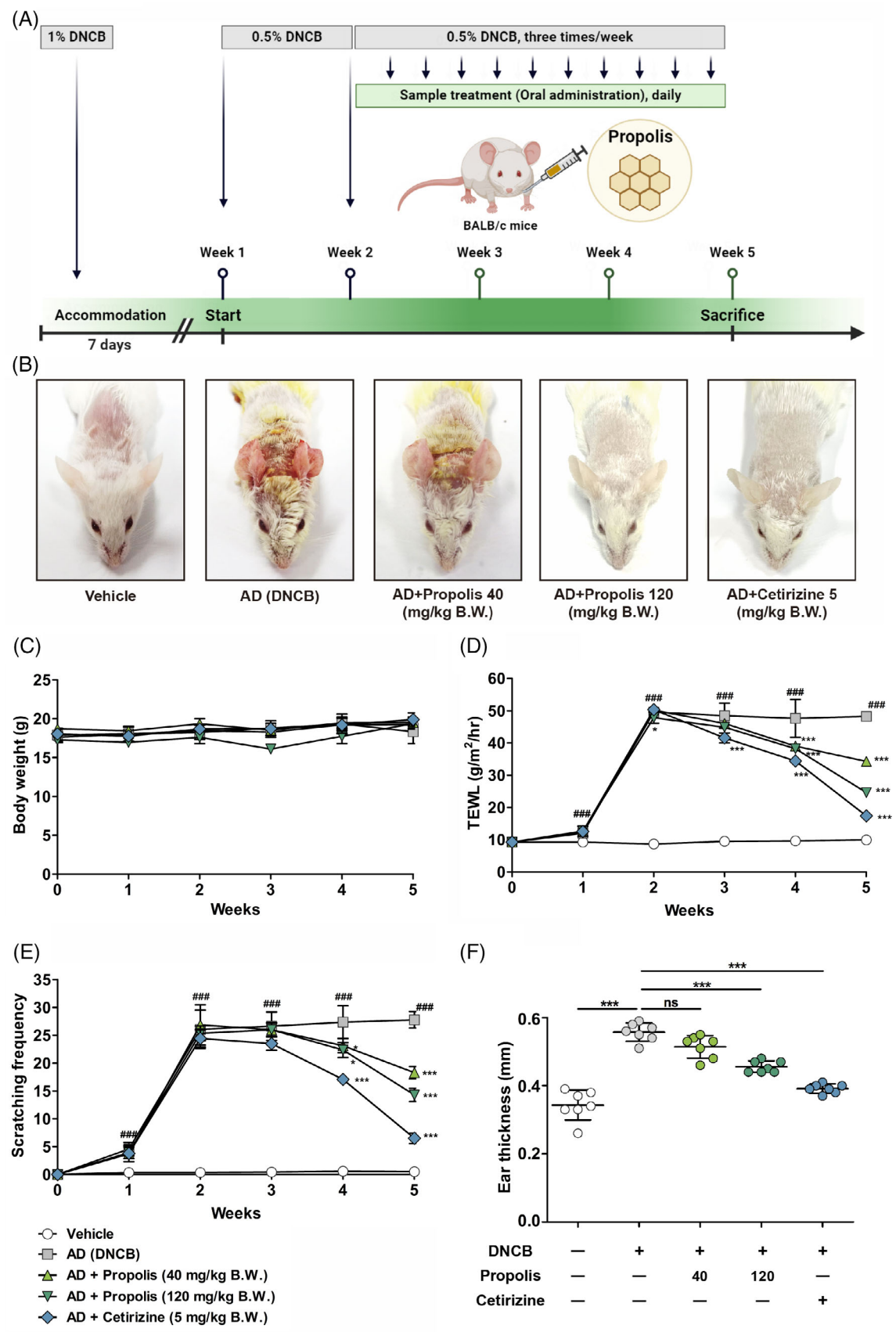


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3.3 | Oral administration of propolis ameliorates AD-like symptoms, skin barrier functions, and pruritus in AD mice

The anti-AD effect of propolis was evaluated using a 2,4-dinitrochlorobenzene (DNCB)-induced BALB/c mouse model, with cetirizine used as a reference drug (Figure 3A). Cetirizine is a second-generation antihistamine and has been widely used to treat AD for ages.³² The topical application of DNCB triggered various AD symptoms in the dorsal skin and ears of mice, including erythema, edema, dryness, and excoriation (Figure 3B). However, when mice were orally administered with propolis at doses of 40 or 120 mg/kg body weight (B.W.) or cetirizine (5 mg/kg B.W.), these symptoms were significantly alleviated (Figure 3B). There were no significant changes in the body weight of the mice with either propolis or cetirizine treatment (Figure 3C). The induction of AD by DNCB substantially increased transepidermal water loss (TEWL), indicating skin barrier dysfunction. However, treatment with propolis and cetirizine effectively reduced TEWL (Figure 3D). Additionally, DNCB-induced mice exhibited frequent scratching behaviors, a characteristic of pruritus. The administration of propolis notably reduced these behaviors (Figure 3E). In addition, DNCB-mediated ear edema was decreased by the administration of propolis (Figure 3F). Collectively, these results demonstrate that the oral administration of propolis can attenuate AD symptoms in mice.

3.4 | Oral administration of propolis reduces hypertrophy of immune organs in DNCB-induced AD mice

To investigate the effects of propolis on regulating hypertrophy of immune organs in DNCB-induced AD mice, we measured the size and weights of the spleen and four types of lymph nodes. AD influences immune system organs due to elevated systemic immune responses.^{33,34} Induction of AD led to an increase in the size and weight of the spleen (Figure S2A,B). However, treatment with propolis resulted in a dose-dependent reduction in the size and weight of the spleen (Figure S2A,B). Similarly, swollen axillary, brachial, cervical, and draining lymph

nodes decreased in size and weight by propolis treatment (Figure S2C–G). Collectively, these results indicate that oral administration of propolis can downregulate the hypertrophy of the spleen and lymph nodes in mice.

3.5 | Oral administration of propolis inhibits AD pathologies in mice skin

Histopathological analysis of the dorsal skin from DNCB-induced mice using H&E staining revealed notable hyperkeratosis and epidermal hyperplasia (Figure 4A). However, treatment with propolis at a dose of 120 mg/kg B.W. resulted in a significant reduction in skin thickness (Figure 4A).

To further determine the regulatory effect of propolis on AD-related biomarkers, we analyzed the dorsal skin of DNCB-induced mice at the molecular level. In the skin of the DNCB-only group, the mRNA levels of Th2 cytokines (IL-4 and IL-13) were overexpressed (Figure 4B,C). However, oral administration of propolis downregulated the expression of these genes (Figure 4B,C). Additionally, the DNCB-induced reduction in filaggrin and involucrin levels was restored by the administration of propolis (Figure 4D,E). Furthermore, the expression of pruritus-inducing cytokines, such as IL-25 and IL-33, was upregulated in the skin of DNCB-induced mice but was reduced by propolis treatment (Figure 4F,G). At the protein expression level, another pruritus-associated cytokine, TSLP, showed enhanced expression in the AD group (Figure 4H). However, propolis treatment downregulated the expression of TSLP in the mice's skin (Figure 4H). Moreover, the application of DNCB led to the inhibition of the skin structural protein, loricrin, but propolis administration restored the expression of loricrin (Figure 4H).

3.6 | Propolis targets the MKK4-p38 signaling pathway

As propolis attenuates AD pathologies and biomarkers in cells, human skin tissues, and mouse models, we sought to investigate the underlying molecular mechanism. MAPK, Akt, and NF- κ B pathways have been known to

FIGURE 3 Effects of propolis on atopic dermatitis (AD) symptoms, barrier dysfunction, and pruritus in 2,4-dinitrochlorobenzene (DNCB)-induced mice. (A) Experimental timeline of AD mouse model (created with BioRender.com). (B) Representative photographs of mice of each group. (C–E) During the experimental period, the effect of propolis on (C) body weight, (D) transepidermal water loss (TEWL), and (E) scratching frequency of mice was determined once a week ($n = 7$). (F) The ear thickness of all mice was measured at the 35th day before sacrificing the mice. Cetirizine served as a positive control (reference drug). Data represent the mean $\pm$ standard deviation (SD).

$p < 0.001$ versus vehicle group. * $p < 0.05$; *** $p < 0.001$ versus AD-induced group.

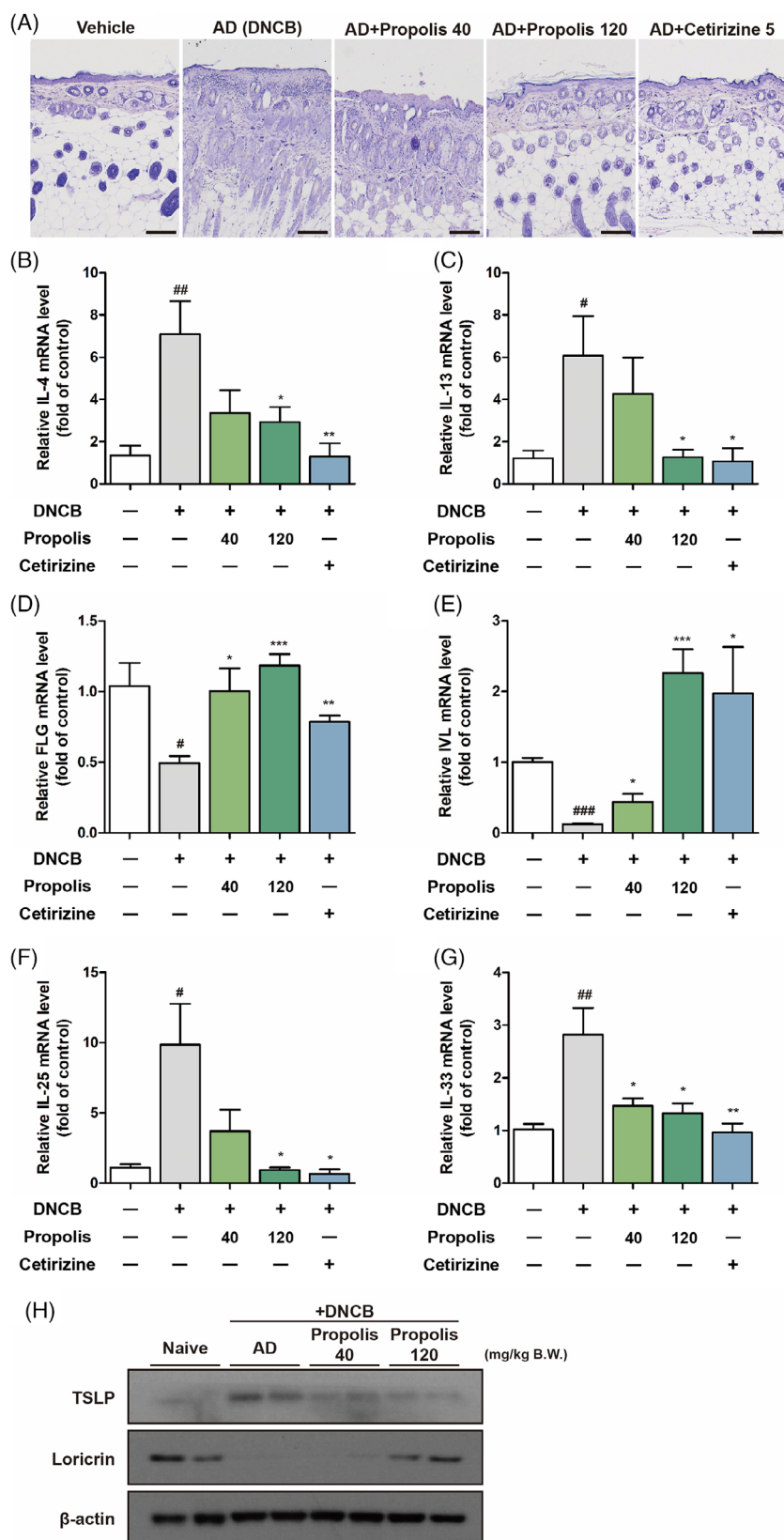
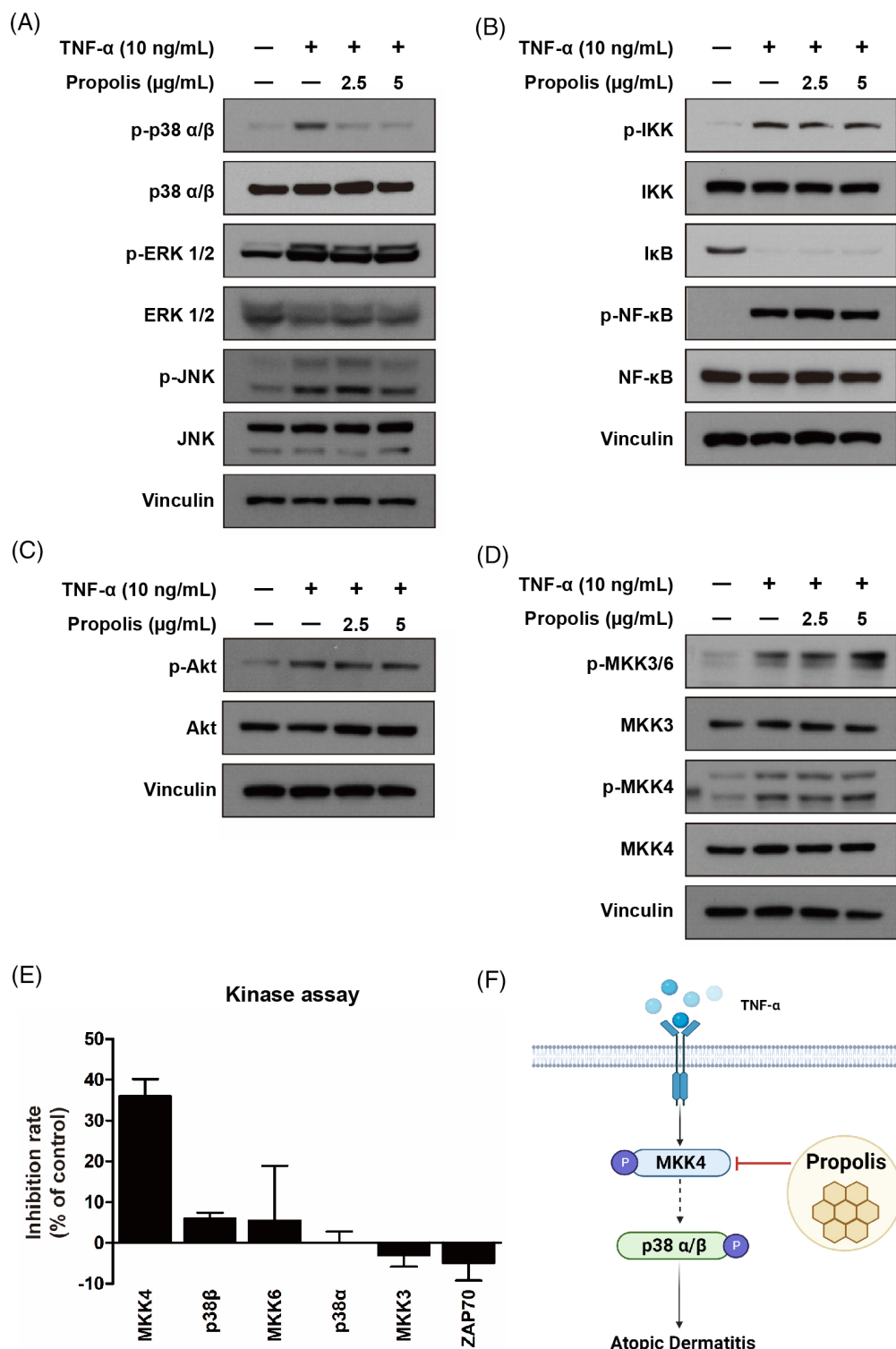


FIGURE 4 Effects of propolis on histopathological symptoms and atopic dermatitis (AD)-related biomarkers in the skin of mice. (A) Representative images of mice dorsal skin tissues stained with hematoxylin and eosin (H&E) (scale bar: 200 μ m). (B–G) Propolis was treated to mice at 40 and 120 mg/kg B.W. and cetirizine was treated at 5 mg/kg B.W. The mRNA expressions of Th2 cytokines, (B) IL-4 and (C) IL-13, barrier proteins, (D) filaggrin (FLG) and (E) involucrin (IVL), and itch-inducing cytokines, (F) IL-25 and (G) IL-33, were examined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Data represent the mean $\pm$ standard error of the mean (SEM). [#] $p < 0.05$; ^{##} $p < 0.01$; ^{###} $p < 0.001$ versus Naïve group, analyzed by t -test. ^{*} $p < 0.05$; ^{**} $p < 0.01$; ^{***} $p < 0.001$ versus AD group, analyzed by t -test. (H) The expression of itch-inducing cytokine (TSLP) and barrier protein (loricrin) was examined using western blotting. β -Actin served as the loading control.

play a key role in AD pathogenesis.^{35,36} Our results revealed that propolis specifically suppressed the phosphorylation of p38 induced by TNF- α , without affecting

the phosphorylation of ERK and JNK (Figure 5A). In addition, propolis did not regulate other inflammation-related signaling pathways, including IKK/NF- κ B and

FIGURE 5 Effect of propolis on MAPK, NF- κ B, Akt, and MAP2K signaling pathways. Effects of propolis on TNF- α -induced (A) MAPK pathway, (B) NF- κ B pathway, and (C) Akt phosphorylation were determined by western blotting in HaCaT keratinocytes. The levels of phosphorylated and basal forms of indicated proteins were examined. (D) The levels of phosphorylated and basal forms of MKK3/6 and MKK4 were examined by western blotting. Vinculin served as the loading control. (E) Kinase activities were determined after propolis treatment. Results are expressed as percentage inhibition of the kinase activity compared to the dimethyl sulfoxide (DMSO) control. (F) The MKK4-p38 signaling pathway is regulated by propolis (created with [BioRender.com](https://www.biorender.com)). MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor kappa B; Akt, protein kinase B; MAP2K, mitogen-activated protein kinase kinase.



Akt (Figure 5B,C). We further examined the effect of propolis on upstream regulators of the p38 pathway, including MAPK kinase (MKK) 3/6 and MKK4. However, propolis did not show any effects on the phosphorylation of both MKK3/6 and MKK4 (Figure 5D). Quantification of the blots is shown in the supplementary file (Figure S3). Thus, we hypothesized that propolis might directly target p38 or its upstream regulators,

leading to the inhibition of p38 phosphorylation. Therefore, we conducted kinase assays against p38 α , p38 β , and its potential upstream kinases, MKK3, MKK4, MKK6, and Zap70 (Figure 5E). The results of the kinase assay showed that propolis exhibited specific inhibitory activity against MKK4, as compared to the other kinases. These results suggest that propolis targets the MKK4-p38 axis (Figure 5F).

3.7 | Quantification of phenolic compounds in propolis extract

To quantify the phenolic compounds present in propolis, a total of 17 compounds were selected as constituents of the propolis extract through previous studies and a comprehensive literature search.^{37–41} For quantitative analysis, corresponding standard compounds for each of the selected 17 compounds were utilized, and their calibration data are presented in Table S2 and Figure S4. Table 1 displays the quantitation results of these 17 compounds present in the propolis extract, arranged in descending order based on their content levels.

3.8 | Identification of active compounds responsible for the anti-AD activity of propolis

To determine the active compound responsible for the anti-AD activity of propolis, a comparative analysis was conducted on the selected compounds out of the 17 identified in the propolis extract, as shown in Table 1. Ten compounds were chosen based on their amount. To assess their effects, HaCaT cells were treated with each of the ten compounds. Among the tested compounds, caffeic acid phenethyl ester (CAPE) showed the strongest inhibitory effect against MCP-1 and IL-8 compared to

other compounds (Figure 6). This observation suggests that CAPE may contribute as a major component in the anti-AD activity of propolis.

3.9 | Caffeic acid phenethyl ester (CAPE) targets the MKK4-p38 signaling pathway

Since CAPE was identified as the major active component responsible for the anti-AD effects of propolis, investigations were conducted to reveal whether CAPE shares a similar mechanism with propolis in regulating the MKK4-p38 signaling pathway. Specifically, CAPE showed a potent inhibitory effect on the activity of MKK4 (Figure 7B) and the phosphorylation of p38 (Figure 7C and Figure S5). In addition, similar to propolis, CAPE did not affect the phosphorylation of MKK3/6 and MKK4 (Figure 7C and Figure S5).

Docking experiments were conducted to explore the interactions between CAPE and MKK4 (Figure 7D,E). The crystal structure of MKK4, identified by its Protein Data Bank (PDB) identifier 3ALO, has been previously reported. MKK4 possesses two active sites, namely the adenosine triphosphate (ATP) binding site and the p38 α binding site. Therefore, we performed separate docking simulations for the ATP binding site (Figure 7D) and the p38 α binding site (Figure S6). The CDOCKER energy calculated for CAPE's binding to the ATP binding site was found to be -42.097 kcal/mol, indicating greater thermodynamic stability compared to the p38 α binding site, where the energy was measured at -36.070 kcal/mol. Furthermore, the interactions discerned between MKK4 and CAPE exhibited a heightened correlation in the ATP binding site than in the p38 α binding site. Several amino acids from the ATP binding site were engaged in interactions with CAPE, including Lys187, Asp229, Lys231, Asn234, and Asp247, through the formation of hydrogen bonds (Figure 7E). Additionally, interactions involved Ile108, Val116, Ala129, and Leu236 through π -alkyl forces, along with Met178 through π -sulfur interactions (Figure 7E). The outcomes of this docking analysis suggest a plausible affinity of CAPE for the ATP binding site of MKK4. While additional structural investigations are warranted to fully comprehend the precise interactions between CAPE and MKK4, our findings strongly indicate that CAPE targets the activity of MKK4, likely through binding to the ATP binding site of MKK4.

4 | DISCUSSION

In the current study, we have demonstrated the therapeutic effect of propolis against atopic dermatitis (AD) using cell, animal, and human skin tissue models. Caffeic acid

TABLE 1 Quantification results of major phenolic compounds included in propolis extracts.

Compound	Content (mg/kg)
3-O-Acetylpinobanksin	78072.80 $\pm$ 697.29
Pinocembrin	44367.60 $\pm$ 1119.25
Quercetin	40133.33 $\pm$ 1262.00
Chrysin	35834.87 $\pm$ 285.08
Caffeic acid	20814.13 $\pm$ 631.78
Galangin	16672.07 $\pm$ 315.27
Pinobanksin	10882.60 $\pm$ 193.14
Caffeic acid phenethyl ester	9077.27 $\pm$ 40.02
p-Coumaric acid	6429.47 $\pm$ 278.44
pinobanksin-5-methyl ether	4414.30 $\pm$ 44.39
Isorhamnetin	2338.43 $\pm$ 79.40
Kaempferol	1818.90 $\pm$ 72.74
trans-Cinnamic acid	1695.30 $\pm$ 83.03
Apigenin	1504.40 $\pm$ 31.29
Acacetin	972.29 $\pm$ 7.26
Naringenin	439.47 $\pm$ 10.06
Gallic acid	272.29 $\pm$ 12.80

Note: All values represent the mean $\pm$ standard deviation (SD).

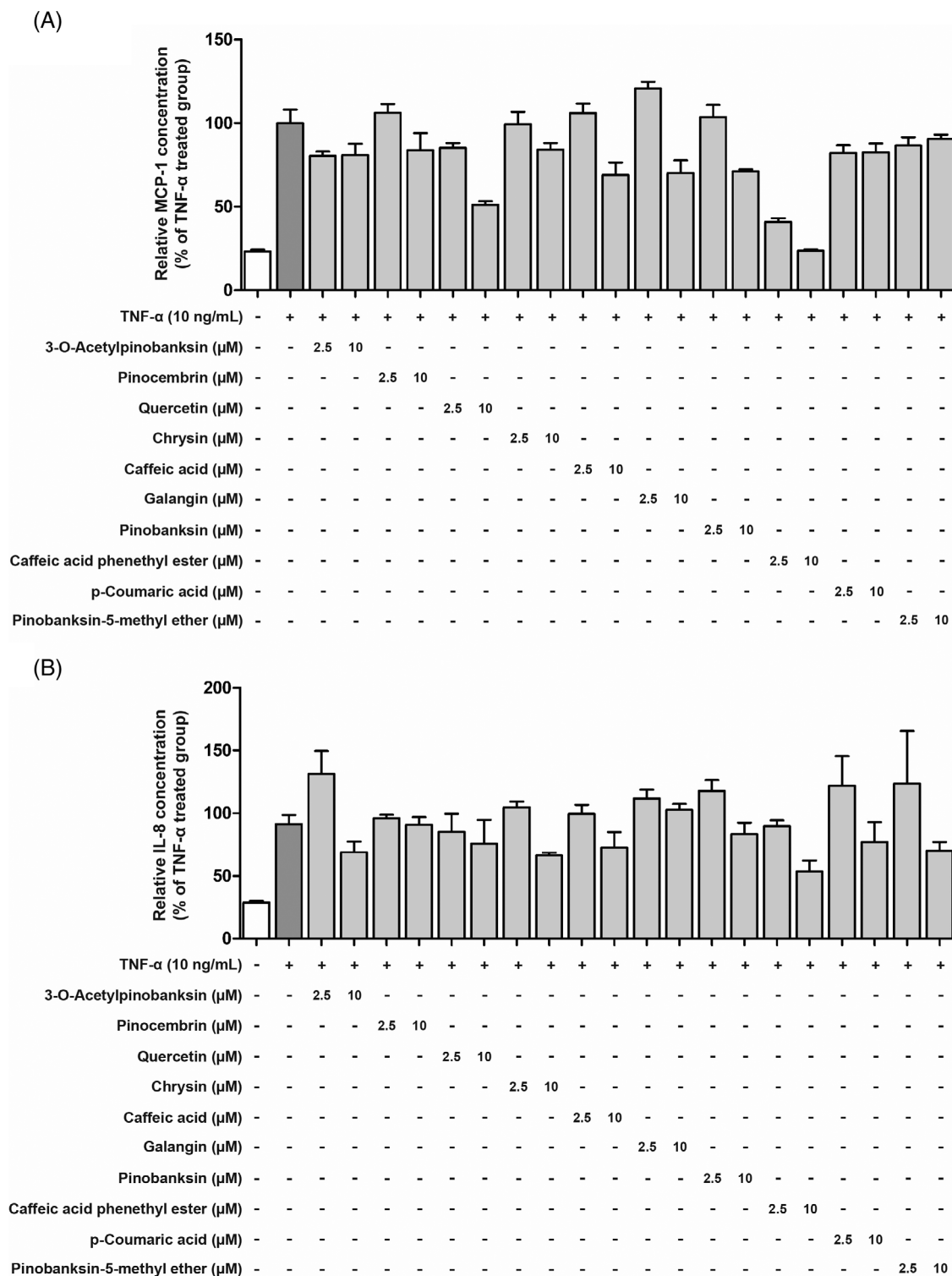


FIGURE 6 Effects of the constituents in propolis extract on the level of atopic dermatitis (AD)-related biomarkers in HaCaT keratinocytes. (A) Cells were treated with the indicated 10 compounds (2.5 and 10 μ M) for 1 h and then TNF- α (10 ng/mL) was added and incubated for 24 h. The concentration of monocyte chemoattractant protein-1 (MCP-1) and IL-8 in culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA) ($n = 3$), and all values are indicated by the percent of the induced group.

phenethyl ester (CAPE) was identified as a major active ingredient of propolis contributing to the anti-AD effect, and MKK4 was discovered as the molecular target of

propolis and CAPE. While propolis was previously reported to regulate several molecular markers of AD in a cell model,⁴¹ this is the first study to show that propolis

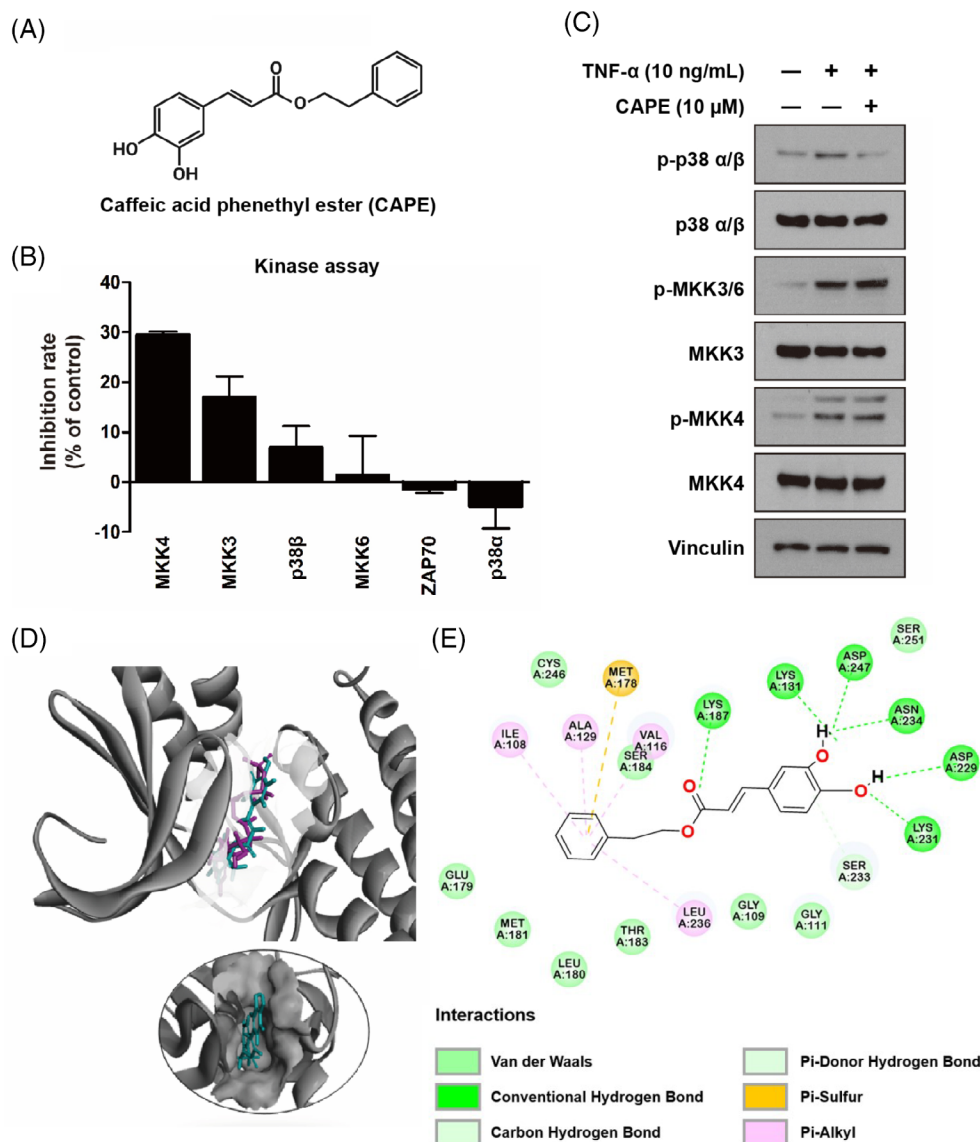


FIGURE 7 Effects of caffeic acid phenethyl ester on the activities of p38 and MAP2K signaling pathway. (A) The molecular structure of caffeic acid phenethyl ester (CAPE). (B) Kinase activities were determined after CAPE treatment (10 μ M). Results are expressed as percentage inhibition of the kinase activity measured in the dimethyl sulfoxide (DMSO) control wells. (C) Effects of CAPE on regulating the phosphorylation of p38 and MKKs were determined by western blotting in HaCaT keratinocytes. Vinculin served as the loading control. (D–E) Docking prediction of CAPE to the MKK4's adenosine triphosphate (ATP) binding site. (D) The binding conformation among CAPE (represented in cyan), Adenylyl imidodiphosphate (AMP-PNP) (represented in purple), and the active site of MKK4 was depicted. (E) The chemical binding residues and interactions were shown in a 2D diagram.

can suppress AD in animal models and to suggest the direct molecular target of propolis responsible for the anti-AD effect.

AD is a multifactorial skin disorder, and its exact pathogenesis remains incompletely understood. Currently, two hypotheses discuss the pathogenesis of AD. One hypothesis suggests that abnormal and localized inflammation acts as a driver, leading to an imbalance in immune response, dysregulation of immune cells, overexpression of cytokines, and subsequent weakening of the skin barrier.³ On the other hand, the second hypothesis proposes that a disrupted epidermal barrier, prone to dehydration and external stimuli, promotes inflammation and triggers systemic immune responses.² Epithelial cytokines such as TSLP, IL-25, and IL-33 contribute to pruritus, leading to scratching behavior and consequent damage to the skin barrier.⁴² These cytokines also initiate T-cell responses within the epidermis, acting as

aggravating factors that reinforce the progressions of AD.⁴² As multiple factors interact and play a role in the pathogenesis of AD, agents that can address various aspects of the disease simultaneously could achieve meaningful responses in AD patients. Our investigations show that propolis can exert diverse therapeutic effects, controlling inflammation, pruritus, and barrier function.

This research investigated the anti-AD activities of propolis by examining its activity using cell and animal models. The concentrations of propolis used ranged from 2.5 to 5 μ g/mL in vitro and ex vivo and from 40 to 120 mg/kg body weight (B.W.) in vivo. Previous studies indicated that a 50–600 mg/kg B.W. daily dose of propolis does not elicit toxic effects.^{43–47} We used relatively low concentrations compared to these reports to avoid potential toxicity and maintain physiological relevance. Multiple studies have used propolis concentrations up to 50 μ g/mL or higher in vitro.^{46,48–50} We tested the



cytotoxicity of propolis in our system, selected noncytotoxic concentrations, and applied the lowest effective concentration (i.e., below 5 $\mu\text{g/mL}$). The current study used CAPE concentrations between 2.5 and 10 μM , whereas many previous studies applied CAPE above 10 μM .^{51–54} These findings suggest that the concentrations used in our study are likely to show therapeutic effects in vivo within a physiologically meaningful range. Propolis showed significant reductions in inflammation mediators and restoration of barrier proteins in keratinocytes. Oral administration of propolis alleviated AD symptoms in mice, even demonstrating that consuming propolis may deliver a beneficial effect on AD patients. Importantly, the efficacy of propolis was also confirmed in a human skin tissue model. This model aimed to mimic AD progression in human skin lesions by treating human skin tissues with TNF- α for 2 weeks. The results showed that propolis treatment inhibited inflammatory biomarkers while promoting skin barrier proteins' recovery. These findings suggest the potential applicability of propolis for human use and provide a foundation for further research and development to prevent AD.

Plant-based substances contain numerous bioactive components, and propolis, in particular, exhibits various compositions due to its characteristics, making it difficult to identify the active component.¹⁹ In this study, we analyzed the contents of 17 phenolic acids and flavonoids present in propolis extract and examined the top 10 compounds among the ingredients to assess their effects on suppressing AD mediators. CAPE exhibited the strongest inhibitory effect against inflammatory cytokine/chemokine production in keratinocytes. According to previous studies, CAPE has anti-inflammatory and immunomodulatory effects in mice.^{55,56} However, the effect of CAPE on AD and its underlying mechanism remained unknown. Therefore, we investigated the mode of action responsible for the anti-AD activities of propolis and its active compound, CAPE. Through molecular analysis, we found that propolis and CAPE selectively inhibit the activity of MKK4. However, the different degrees of kinase inhibition exerted by propolis and CAPE, particularly concerning MKK4 and MKK3, imply a complex and potentially redundant interplay of multiple components that remains to be identified. MKK4 functions as an upstream kinase protein that regulates the phosphorylation of p38 in the MAPK pathway. The p38 pathway plays a role in keratinocyte differentiation for general protection as well as the regulation of apoptosis during stressful situations.⁵⁷ Inhibiting p38 has been shown to suppress the Th2 immune response in AD mice and restore skin barrier defects in mice and keratinocytes.^{58,59} Therefore, the anti-AD activity of propolis and CAPE is at least partially attributed to its inhibition of

the MKK4-p38 axis. Furthermore, the modeling data supports that CAPE would inhibit the activity of MKK4 by binding to the ATP binding site of MKK4. Based on these results, we propose that CAPE serves as a major active compound responsible for the anti-AD and MKK4 inhibitory effects exerted by propolis.

In summary, our study demonstrates that propolis exhibits anti-inflammatory, skin barrier-enhancing, and antipruritic effects in the cell, human skin, and mice models. The results from cell and human skin models indicate that propolis possesses beneficial functions, including inhibiting inflammatory biomarkers and promoting the recovery of skin barrier proteins. In vivo findings further validate the effects of propolis by regulating the scratching behaviors, ameliorating AD symptoms, and restoring the skin barrier in mice. These effects are mediated by targeting MKK4 in the p38 signaling pathway and are achieved through the active compound, CAPE. Our findings highlight the potential and mechanisms of Korean propolis as a natural therapeutic agent for the treatment of AD.

AUTHOR CONTRIBUTIONS

Conceptualization, Y.R.C., G.A., and S.B.; Methodology, Y.R.C., E.J.H., E.H., A.M.K.J., J.W., S.L., and T.K.; Validation, Y.R.C.; Investigation, Y.R.C., E.J.H., E.H., A.M.K.J., J.W., S.L., T.K., and S.K.K.; Resources, S.O.W., and G.H.; Writing—Original Draft, Y.R.C., and S.B.; Editing, Y.R.C., E.H., and S.B.; Supervision, S.L., G.H., W.K., G.A., and S.B.; Project Administration, G.A., and S.B.; Funding Acquisition, S.B. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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